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Effect of bacteriorhodopsin on the orientation of the headgroup of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine in bilayers: a ^{31}P- and ^{2}H-NMR study

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Bacteriorhodopsin (BR), purified from the halophilic bacterium, *Halobacterium halobium*, has been separated from the endogenous purple membrane lipids and reconstituted by detergent dialysis into bilayers of the zwitterionic phospholipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), which was selectively deuterated at the headgroup in the choline α- and β-methylene segments and the choline γ-methyl groups. Complexes of DMPC/BR contents from 67:1 to 222:1 (mol/mol) were produced under conditions to promote formation of large vesicles (mean diameters 600–700 nm). The magnitudes of the ^{2}H-NMR quadrupole splittings recorded from the deuterium-labelled headgroup segments, and the ^{31}P-NMR chemical shift anisotropy (CSA) of the phosphate group appeared to vary linearly with the BR content in the complexes over the range of DMPC/BR ratios studied. On increasing the proportion of BR in the DMPC-BR complexes, the ^{2}H-NMR quadrupole splittings measured from the choline γ-methyl groups and the β-methylene segments and the ^{31}P-NMR CSA increased in magnitude, while the ^{2}H-NMR quadrupole splitting from the α-methylene segment decreased. Such opposing changes in the choline α- and β-methylene segment quadrupole splittings are similar to those reported on increasing the proportion of positively charged amphiphile at the bilayer surface (Seelig et al. (1987) Biochemistry 26, 7535–7541). It is suggested that BR presents a net positive charge to the phosphocholine headgroups at the protein/lipid interface.

Introduction

Purple membrane (PM), purified from the halophilic bacterium *Halobacterium halobium*, contains a single species of integral membrane protein, bacteriorhodopsin (BR), of molecular weight 26 000 [1]. The polypeptide chain of the BR molecule is arranged into seven, closely packed, α-helical segments which extend approximately perpendicular to the plane of the membrane [2,3] and one molecule of retinal is covalently linked to each 26 kDa BR molecule [1]. PM is unusual in its low lipid content (20% by weight [4]). The endogenous PM polar lipids are acidic and the major phospholipid species, 1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoryl-3′-*sn*-glycerol 1′-phosphate (DPhPGP), has the potential to bear three negative charges [4], which may aid interaction of the lipids with the BR. Studies of the aggregation properties of BR in reconstituted phosphatidylcholine (PC) bilayer systems [5,6], suggest that the endogenous PM lipids are important in mediating aggregation of the protein particles into a hexagonal two-dimensional lattice similar to that observed in PM [7,8]. The spectral properties of the retinal chromophore itself are influenced by lipid charges at the membrane surface [9]. Thus, ionic interactions and charge distributions between the BR and the lipid headgroups at the bilayer membrane surface may be

Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPhPGP, *H. halobium* 1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoryl-3′-*sn*-glycerol 1′-phosphate; DPhPGS, *H. halobium* 1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoryl-3′-*sn*-glycerol 1′-sulphate; SDS, sodium dodecyl sulphate; $\Delta\nu_Q$, quadrupole splitting; CSA, chemical shift anisotropy; FID, free induction decay; T_m, mid-point temperature for bilayer main gel to liquid-crystalline phase transition.

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important in influencing the properties of the BR, although the bacterium grows in high salt where global surface charge effects may be screened.

Here the effect of BR content on the motional and conformational properties of the phosphocholine headgroups at the membrane surface of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers has been investigated by broadline phosphorus-31 and deuterium nuclear magnetic resonance (NMR). The deuterium quadrupole splittings ($\Delta\nu_q$) measured from broadline ^{2}H-NMR spectra of PC bilayers, in which segments within the phosphocholine headgroup have been specifically labelled with deuterium, are sensitive to the electric charge at the surface of the membrane [10]. To permit the effect of BR on the bilayer surface charge to be assessed, the endogenous PM lipids have been completely removed from the BR (confirmed by high resolution ^{31}P-NMR [6]) using molecular exclusion chromatography [11] prior to reconstitution by detergent dialysis with DMPC. The DMPC used to produce the bilayer complexes was selectively deuterated at either the three γ-methyl groups (DMPC-d_9), or the α- and β-methylene segments (DMPC-d_4) of the choline headgroup. Deuterium and phosphorus-31 NMR spectra thus provide information on all segments of the phosphocholine headgroup. The measured ^{2}H-NMR quadrupole splittings and the ^{31}P-NMR chemical shift anisotropies (CSA) provide structural information on the time-averaged amplitude of motion or the conformation, or both, of the particular segment within the phospholipid headgroup [12,13].

Materials and Methods

Phospholipid synthesis. The phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was specifically deuterated at the N,N,N-trimethyl moiety of the choline headgroup by methylation of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) using CD_3I to produce DMPC-d_9 [14]. DMPC-d_4, specifically deuterated in the choline Cα and Cβ headgroup methylene segments, was produced from dimyristoylglycerol using per-deuterated ethanolamine as described previously [15]. After purification by HPLC and recrystallization from acetone, both lipids ran as single species on thin-layer chromatography with $CHCl_3$/methanol/ammonia 65:30:3, v/v/v).

Production of DMPC-bacteriorhodopsin complexes free of endogenous purple membrane lipids. DMPC-d_4 and DMPC-d_9 were mixed in the ratio 4:1 (w/w) prior to reconstitution with BR by dialysis. The method adopted for production of DMPC-BR complexes and analysis of the DMPC-BR contents (mol/mol) has been described [6]. For all the DMPC-BR complexes studied in this work, the rate of cholate removal prior to vesicle formation during reconstitution by dialysis from cholate-solubilized BR and DMPC was controlled by inclusion of 0.1–0.2% (w/w) cholate in the dialysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.025% azide). Once vesicles were judged to have formed, usually after 4–5 days, dialysis was performed without cholate in the buffer and in the presence of Amberlite XAD-2 beads (BDH, Poole, UK), which had previously been washed three times in acetone and boiled in distilled water with frequent changes. Dialysis was continued for 4–5 days with twice daily changes of buffer. After purification by linear sucrose density gradient centrifugation [6], the BR-containing band was washed free of sucrose in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) (three times; 30 min; 15°C; 100000 × *g*), washed twice from the same buffer but made from deuterium-depleted water (Sigma) and then the pellet loaded into an NMR tube.

Broad line nuclear magnetic resonance. Broad line ^{2}H-NMR and ^{31}P-NMR spectra of DMPC(-d_4/-d_9)-BR complexes were recorded on a Nicolet (360 MHz for ^{1}H) spectrometer at 55.3 MHz and 145.9 MHz, respectively. Deuterium NMR spectra were collected into 2K points after a pre-acquisition delay of 30 μs with a sweep width of 40000 Hz. ^{2}H-NMR spectra of DMPC-d_9-BR complexes were recorded at 46.1 MHz on a Bruker WH300 NMR spectrometer using a sweep width of 10000 Hz. A relaxation delay of 250 ms was used. The FIDs were zero-filled to 8K and a line broadening of 40 Hz applied with exponential multiplication. The apparent quadrupole splittings ($\Delta\nu_q$) were measured from the turning points of the powder pattern, as judged by the point of greatest height. Phosphorus-31 NMR spectra ($\pi/2$ pulse width, 18 μs) were collected into 4K points after a pre-acquisition delay of 40 μs with a sweep width of 40000 Hz. The FIDs were zero filled to 8K and a line broadening of 80 Hz applied with exponential multiplication. A relaxation delay of 1 s was used. The chemical shift anisotropy (CSA) values (ppm) were measured from the half-heights of the upfield (90° orientation) and downfield (0° orientation) intensities. ^{31}P-NMR spectra were not proton decoupled.

Determination of vesicle size by negative stain electron microscopy. Vesicle suspensions (appropriately diluted with 10 mM Tris-HCl, 1 mM EDTA (pH 7.5)) were stained with 2% uranyl acetate and electron microscopy performed on a Philips 400 electron microscope with normalization at a magnification of 28000. For each lipid-protein complex at least five photographs were taken to ensure representative sampling. The diameters of all vesicles recorded on each negative were recorded and included in the statistical assessment.

Results

A total of nine complexes of DMPC/BR (mol/mol) ratios ranging from 67:1 to 379:1 were produced. Of these, three (DMPC/BR contents 68:1, 131:1, and 141:1 mol/mol) were produced with DMPC-d_0 alone and five (DMPC/BR contents 67:1, 95:1, 182:1, 218:1 and 222:1, mol/mol) were produced with both DMPC-d_4 and DMPC-d_0. Linear sucrose density gradients of these complexes displayed a single sharp band. As a control, a mixture of the 182:1 and 222:1 (mol/mol) DMPC-BR complexes was resolved into two separate bands on a 10–30% (w/w) linear sucrose density gradient, while each complex alone produced a single sharp band (~ 1 mm width) demonstrating the vesicles comprising these complexes to be of homogeneous protein/lipid content.

Negative stain electron microscopy studies showed the complexes to be comprised of large vesicles with arithmetic mean diameters between 600 and 700 nm and some vesicles measuring over 2000 nm in diameter. Statistical parameters calculated for diameters of vesicles comprising three of the DMPC-BR complexes are presented in Table I.

Solid-state ^2H-NMR

In Figs. 1a–1d ^{2}H-NMR spectra recorded at 45°C for DMPC($-d_4/-d_0$)-BR complexes of lipid/protein ratios ranging from 67:1 to 222:1 (mol/mol) are shown. The ^{2}H-NMR spectrum of protein-free DMPC-d_4 dispersions is shown in Fig. 1e. The outer powder pattern is assigned to the choline α-CD_2 segment [15,16] and for protein-free DMPC-d_4 bilayers consists of two components of different quadrupole splitting (Fig. 1e) as also shown for DPPC bilayers [17] and observed for phosphatidylserine [18] and phosphatidylglycerol bilayers [19]. In the presence of BR, the two signals from the α-CD_2 segment are virtually unresolved and a broad powder pattern is observed (Figs. 1c, 1d). The inner powder pattern in the spectrum from protein-free DMPC-d_4 dispersions (Fig. 1e) is assigned to the choline β-CD_2 segment [16]. The central powder pattern, with quadrupole splittings of approximately 1 kHz, in spectra from the DMPC($-d_4/-d_0$)-BR complexes (Figs. 1a–1d) thus arises from the choline γ-CD_3 groups.

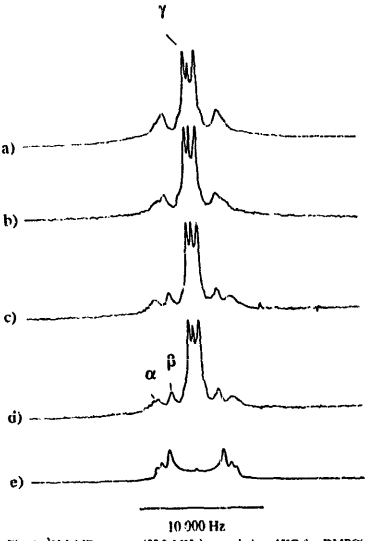

Fig. 1. ^{2}H-NMR spectra (55.3 MHz) recorded at 45°C for DMPC($-d_4/-d_0$)-BR complexes of DMPC/BR (mol/mol) contents, 67:1 (a); 95:1 (b); 182:1 (c); and 222:1 (d). The spectrum of protein-free DMPC-d_4 dispersions (e) is presented for comparison. All endogenous purple membrane lipids were removed from complexes and samples contained between 50 and 70 mg DMPC (DMPC-d_4/DMPC-d_0 ratio 4:1, w/w) in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5).

TABLE I

Statistics for diameters of vesicles comprising DMPC-BR complexes

DMPC/BR (mol/mol)	Diameter		Number measured	Diameter range (nm)
	mean (nm)	standard deviation (nm)		
68:1	646	355	74	150–1500
141:1	699	320	61	100–1200
379:1	686	522	24	150–2200

Effect of temperature on ^2H-NMR powder patterns from the choline α- and β-CD_2 segments in DMPC-BR complexes

In Fig. 2, the measured quadrupole splittings from the choline α- and β-CD_2 segments in complexes with DMPC/BR ratios of 67:1, 95:1 and 182:1 (mol/mol) together with those recorded from protein-free DMPC dispersions are plotted as a function of temperature. The quadrupole splitting used for the αCD_2 segment for protein-free DMPC dispersions is an average of the two component powder patterns (Fig. 1e). This was judged to approximate best the values obtained for the DMPC-BR complexes, in which the two individual splittings are unresolved in the broad powder pattern. In the ^{2}H-NMR spectrum of protein-free DMPC at

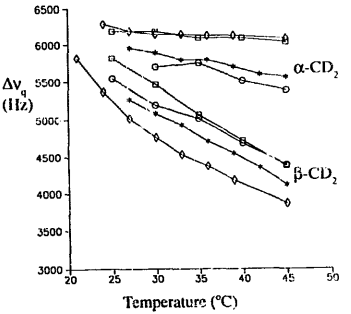

Fig. 2. ^{2}H-NMR quadrupole splittings (recorded at 55.3 MHz) for the choline α- and β-CD_2 segments in bilayer complexes of DMPC/BR (mol/mol) ratios, 67:1 (○), 95:1 (*) and 182:1 (◇), compared to those for protein-free DMPC dispersions (□) as a function of temperature.

25°C (not shown), the α- and β-CD_2 powder patterns are not well resolved. On warming the sample further above the main gel to liquid-crystalline phase transition, the α-CD_2 segment quadrupole splittings do not vary significantly with values of ~6 kHz, while those for the choline β-CD_2 segment decrease [15,16] and the β-CD_2 segment powder pattern becomes clearly resolved from that of α-CD_2 segment (Fig. 1e). The quadrupole splittings recorded from the choline α- and β-CD_2 segments in the presence of BR exhibit similar temperature dependent trends to those of protein-free DMPC dispersions, with the β-CD_2 segment quadrupole splittings being temperature sensitive and the α-CD_2 segment quadrupole splittings remaining virtually independent of temperature. However, the absolute magnitudes of both α- and β-CD_2 segment quadrupole splittings are influenced by the protein content of the bilayer (as described below).

Effect of BR content on the ^{2}H-NMR powder patterns from the choline α- and β-CD_2 segments and γ-CD_3 groups

^{2}H-NMR powder patterns from the choline α- and β-CD_2 segments appear to be sensitive to the BR content at temperatures above T_m (Figs. 1a–d). For protein-free DMPC-d_4 dispersions (Fig. 1e) and the lower protein content DMPC-BR complexes (Figs. 1c and d), the resonances from the 90° orientation in the α- and β-methylene segment powder patterns are well separated at temperatures above completion of the main gel to liquid-crystalline phase transition. However, increasing the BR content within the DMPC bilayers appears to promote spectral overlap of the resonances from the 90° orientations in the α- and β-methylene segment powder patterns and for the high BR content 67:1 (mol/mol) DMPC-BR complex, the α-CD_2 quadrupole splitting is only just distinguishable under the β-methylene segment powder pattern (Fig. 1a).

The quadrupole splittings for the choline α- and β-CD_2 segments and the γ-methyl groups measured from the spectra of DMPC-BR complexes at two temperatures above T_m (30°C and 45°C) are plotted as a function of the reciprocal of the DMPC/BR (mol/mol) content in Fig. 3. The quadrupole splittings measured for the choline α- and β-CD_2segments and the γ-methyl groups from protein-free DMPC dispersions at 30°C and 45°C are also shown in Fig. 3. Incorporation of small amounts of BR (DMPC/BR ratios, 222:1 to 182:1, mol/mol) into DMPC bilayers causes a significant reduction in the magnitude of the choline β-CD_2 segment and γ-methyl group quadrupole splittings relative to those measured from protein-free DMPC bilayers, while the quadrupole splittings from the choline α-CD_2 segments are relatively unaffected.

Increasing the BR content within the DMPC bilayers induces opposing effects on the magnitudes of the quadrupole splittings measured for the choline α- and

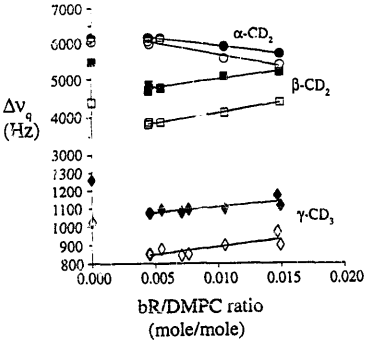

Fig. 3. ^{2}H-NMR quadrupole splittings measured for the choline α-CD_2 (○), β-CD_2 (□) segments and the γ-CD_3 groups (◇) of DMPC in DMPC/BR bilayer complexes as a function of the BR/DMPC content (mol/mol) at temperatures above the main gel to liquid-crystalline phase transition. Filled symbols represent values recorded at 30°C and open symbols those at 45°C. The $\Delta\nu_q$ scale for the γ-CD_3 groups is 10-times larger than for the α- and β-CD_2 segments. All quadrupole splittings recorded at 55.3 MHz except for those of the γ-methyl groups of the 141:1, 131:1 and 68:1 (mol/mol) DMPC-d_4/BR complexes which were recorded at 46.1 MHz.

β-CD_2 segment powder patterns. Thus, the magnitude of the quadrupole splitting for the choline α-CD_2 segment is reduced considerably (Fig. 3) on increasing the BR content of the vesicles, while the choline β-methylene segment quadrupole splitting demonstrates an increase in magnitude. From Fig. 3 it appears that over the range of lipid/protein ratios examined (from DMPC/BR contents of 222:1 to 67:1, mol/mol), the quadrupole splittings measured from the choline α- and β-CD_2 segment powder patterns appear to exhibit a linear dependence on the BR content. The slopes of the best fit line for the variation of the choline β-CD_2 segment quadrupole splitting with BR content for the DMPC-BR complexes were +0.448 and +0.535 kHz per 0.01 BR/DMPC (mol/mol) units at 30°C and 45°C, respectively, while that for the α-CD_2 segment quadrupole splitting was −0.655 kHz per 0.01 BR/DMPC (mol/mol) units at 45°C.

The quadrupole splittings recorded for the choline γ-methyl groups at temperatures between 30°C and 45°C appear to increase linearly in magnitude with BR content (Fig. 3) although the points were scattered about the best fit lines. The correlation coefficient calculated for the variation of the γ-methyl quadrupole splittings recorded at 45°C with BR content over the range of DMPC/BR (mol/mol) ratios 222:1 to 67:1 (Fig. 3) was +0.81, which is statistically significant. A linear dependence of the quadrupole splittings measured from the choline γ-methyl groups with protein/DMPC-d_0 content has been suggested for other integral membrane proteins (band-3 [20] and rhodopsin [21]), although the magnitudes of the quadrupole splittings were observed to decrease with increasing protein content in these cases.

Broadline phosphorus-31 NMR

Broadline phosphorus-31 NMR spectra recorded for DMPC-BR complexes of ratios (mol/mol) 67:1, 95:1, 182:1, 218:1 and for protein-free DMPC dispersions are presented in Fig. 4. All the spectra, irrespective of protein content are seen to be single component and characteristic for phospholipids in a bilayer conformation [22]. There is no evidence of a central narrow spectral component, indicative of phospholipids undergoing isotropic motion, possibly in small vesicles or inverted micelles [22,23]. The spectra from protein-free DMPC dispersions and the BR-containing complexes exhibit varying degrees of macroscopic alignment of the lipid bilayer membranes in the applied magnetic field (spectrometer field strength 8.4 Tesla). The diamagnetic anisotropy of the myristoyl chains orient the DMPC molecules in strong external magnetic fields such that the plane of the bilayers are aligned parallel to the magnetic field [24]. This orientational behaviour is reflected in the spectrum of protein-free DMPC dispersions (Fig. 4e) which displays increased intensity

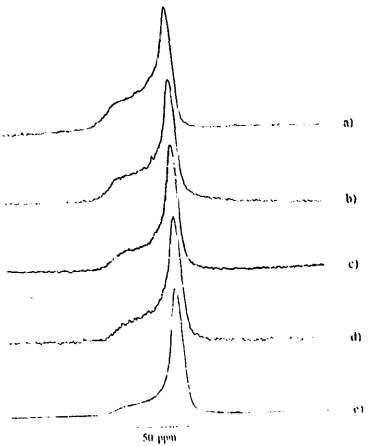

Fig. 4. ^{31}P-NMR spectra (145.9 MHz) recorded at 33°C for DMPC-BR complexes of DMPC/BR (mol/mol) contents, 67:1 (a); 95:1 (b); 182:1 (c); and 218:1 (d), and for protein-free DMPC dispersions (e). All endogenous purple membrane lipids were removed from the complexes and samples contained between 50 and 70 mg DMPC in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5).

for the 90° orientation relative to the 0° orientation compared to a random, spherically averaged powder pattern [24]. Similar effects are observed in the ^{2}H-NMR spectrum of protein-free DMPC dispersions (Fig. 1e). Increasing the BR content (Figs. 4d to 4a) tends to oppose this macroscopic alignment of the lipid bilayers as judged by the increase in intensity of the 0°-orientation component relative to that of the 90°-orientation component when compared to the powder pattern from the protein-free DMPC dispersions. Indeed, the powder patterns from the higher BR content DMPC-BR complexes (Figs. 4a and 4b) resemble more closely that from a spherically averaged system in terms of relative intensities of the 0° and 90° spectral orientations. This may result from the interaction of the net dipole moment from the seven α-helices in the BR molecule with the applied magnetic field [25] in such a manner as to oppose the macroscopic alignment of vesicles arising from the diamagnetic anisotropy of the fatty acyl chains. In PM the α-helices are aligned perpendicular to the plane of the bilayer [2] and in the ^{31}P-NMR powder pattern obtained from PM itself [26,27], the intensity of the 0°-orientation is increased relative to that from the 90°-orientation when compared to a completely spheri-

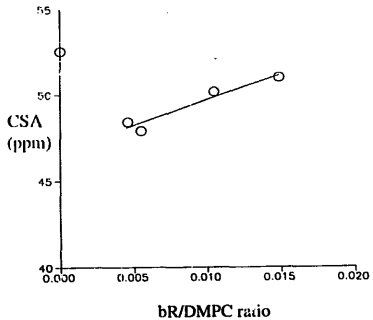

Fig. 5. ^{31}P-NMR chemical shift anisotropies (CSA) measured for the phosphate group of DMPC in DMPC-BR bilayer complexes at 33°C plotted as a function of BR/DMPC (mol/mol) content.

cally averaged bilayer system. The orientation of PM in an applied magnetic field is dependent on the concentration of PM in the suspension [26].

The magnitudes of the CSAs measured from four of the DMPC-BR complexes at a temperature of 33°C are plotted as a function of BR content in Fig. 5, together with the CSA for protein-free DMPC dispersions. Incorporation of small proportions of BR (DMPC/BR contents 218:1 to 182:1, mol/mol) into the DMPC bilayer induce a considerable decrease in magnitude of the CSA relative to protein-free DMPC dispersions. On increasing the BR content, the CSA appears to follow a linear increase in magnitude, reflecting the trends observed for the ^{2}H-NMR quadrupole splittings measured from the choline γ-methyl groups and the β-CD_2 segments (Fig. 3). The change in magnitude of the measured CSA on varying the DMPC/BR content between 182:1 and 67:1 is 6%, which is too large to be attributed to experimental error.

Discussion

Here, DMPC-BR complexes of a range of lipid/protein ratios have been produced by Triton X-100 solubilization and cholate dialysis. High resolution ^{31}P-NMR performed after solubilization of the 67:1, 68:1, 95:1, 131:1 and 222:1 (mol/mol) DMPC-BR complexes in 4% SDS revealed complete removal of the major *H. halobium* PM phospholipid, DPhPGP, and demonstrated DMPC to be the only phospholipid present [6]. In addition, high resolution ^{31}P-NMR spectra of SDS-solubilized DMPC-BR complexes confirmed the integrity of the DMPC component in that no traces of lysophosphatidylcholine [28] or other phosphate-containing species arising from degradation of DMPC were detectable. Freeze fracture electron micrographs of the DMPC-BR complexes used in this study [6] after quenching from temperatures well above completion of the main gel to liquid-crystalline phase transition reveal that the BR particles, possibly trimers, are dispersed; there being no evidence for changes in the protein aggregation state with BR content over the lipid/protein ratios studied.

The DMPC-BR complexes used in this study were comprised of large vesicles. Slowing the rate of vesicle formation in the reconstitution mixture by inclusion of cholate in the dialysis buffer, promotes the generation of much larger vesicles than if cholate removal rates are not controlled prior to vesicle formation (unpublished observations). From the frequency distributions of vesicle diameters measured from freeze-fracture electron micrographs it was estimated (from the vesicle surface areas) that over 80% of the DMPC was accommodated in vesicles with diameters greater than 600 nm. In such large vesicles, the residual spectral anisotropy in broadline ^{2}H-NMR powder patterns will be relatively little influenced by motional averaging resulting from rapid phospholipid reorientation in the applied magnetic field due to lateral diffusion around small vesicles and vesicle tumbling motions [23,29].

The small magnitude of the quadrupole splitting ($\sim$ 1025 Hz at 45°C) observed for the choline γ-methyl groups in DMPC-d_9 bilayers [15] and DPPC bilayers [16] at temperatures above T_m imply extensive motion within this region of the choline headgroup. The increase in quadrupole splitting for segments located nearer the glycerol backbone (4380 Hz for the β-CD_2 and 6040 Hz for the α-CD_2 segments of DMPC at 45°C) reflects a progressive reduction in motional freedom or a different conformation within the choline headgroup [16]. The magnitudes of the quadrupole splitting from the choline α-CD_2 segment in protein-free bilayers of DMPC [15] or DPPC [16] exhibit little variation on increasing the temperature above T_m, while those from the β-CD_2 segment and the γ-methyl groups exhibit a considerable reduction in magnitude. These observations are interpreted in terms of oscillations of the C_γ-$C\beta$ region of the choline headgroup around the $C\alpha$-$C\beta$ bond which increase in amplitude with temperature, while the α-CD_2 and phosphate segments remain relatively rigid [16]. In the presence of BR, the temperature dependencies of the quadrupole splittings for both the α- and β-CD_2 segments (Fig. 2) and the γ-methyl groups (data not presented) of the choline headgroup parallel those from protein-free DMPC bilayers (although the actual quadrupole splitting values vary with BR content). It would appear that the tem-

perature dependence of the motional properties of segments within the phosphocholine headgroup of the bulk bilayer DMPC is preserved in the presence of BR.

Incorporation of small amounts of BR (DMPC/BR contents 222:1 to 182:1, mol/mol) causes a decrease in the magnitudes of ^{2}H-NMR quadrupole splittings from the choline β-CD_2 segment and γ-CD_3 groups and the ^{31}P-NMR CSA measured from the phosphate moiety, relative to protein-free lipid bilayers (Figs. 3 and 5). Similar effects on quadrupole splitting and CSA have been observed for other integral membrane proteins eg sarcoplasmic reticulum Ca^{2+}-ATPase [30], cytochrome-c oxidase [31], band-3 [20] and rhodopsin [21] in PC bilayers, and for the peripheral membrane protein, myelin basic protein, in DMPG bilayers [32] and presumably reflect perturbations in molecular motions or orientation within the phospholipid headgroup at the membrane surface due to accommodation of the uneven surface of the protein.

A linear variation of ^{2}H-NMR quadrupole splitting from headgroup-deuterated lipids with protein/lipid content has been suggested for band-3 [20] and rhodopsin [21] in DMPC bilayers and myelin basic protein in DMPG bilayers [32]. The opposing linear changes in magnitude of the quadrupole splittings from the choline α- and β-CD_2 segments on increasing the BR content in the DMPC/BR bilayers at constant temperature (Fig. 3) reflect those observed on increasing the proportion of positively-charged amphiphiles in POPC bilayers [10]. The dipole moment resulting from the zwitterionic nature of the phosphocholine headgroup is estimated to be 19 D [33]. From X-ray diffraction studies of hydrated PC bilayers [34] the net dipole moment perpendicular to the plane of the membrane is $\sim$ 6.5 D. Such a dipole moment would interact with charged groups on lipids or proteins at the membrane surface resulting in a reorientation of the dipole and hence a change in average amplitude of segmental motion or conformation within the phosphocholine headgroups. Increasing the proportion of positively charged amphiphile at the PC bilayer surface will tend to reorient the phosphocholine dipole such that the positively-charged trimethylammonium moiety is repelled towards the bilayer normal and the negatively-charged phosphate moiety may interact with the positive charges.

With mole fractions of positive or negative charged amphiphiles up to 0.3, the quadrupole splittings from the choline α- and β-CD_2 segments are reported to exhibit a linear variation with mole fraction of amphiphile [10]. Incorporation of the positively charged amphiphile, didodecyldimethylammonium bromide into POPC bilayers to a mole fraction of 0.3, effects a decrease in the choline α-CD_2 quadrupole splitting by 8.27 kHz (as measured from Fig. 4 of Seelig et al. (1987) [10]). Increasing the mole fraction of BR in DMPC-BR complexes by 0.01 BR/DMPC (mol/mol) units over the range of DMPC/BR contents studied (222:1 to 67: DMPC/BR (mol/mol)) results in a 0.655 kHz reduction in the magnitude of the α-methylene quadrupole splitting measured at 45°C (best fit line, Fig. 3). Such a reduction in magnitude of the α-CD_2 segment quadrupole splitting is equivalent to incorporation of the didodecyldimethylammonium ion to a mole fraction of 0.024 in POPC bilayers. This suggests the net positive charge presented by BR at the lipid/protein interface on the membrane surface is 2.4-times that for the didodecyldimethylammonium ion assuming that contributions to perturbation of the phosphocholine headgroup from steric effects of the BR surface are similar to those from the didodecyldimethylammonium ion. The BR molecule, however, is a transmembrane protein and interacts with the headgroups of lipids in both leaflets of the bilayer. In contrast, each didodecyldimethylammonium ion occupies only one leaflet of the bilayer at any one time. Thus the net perturbation of the phosphocholine headgroups by BR is distributed over two membrane surfaces and in terms of the total electric charge presented by the protein, each molecule of BR may be equivalent to 4.8 didodecyldimethylammonium ions. Furthermore, the BR molecule presents a larger surface area to the DMPC than a didodecyldimethylammonium ion and thus the perturbation of the phosphocholine groups by positive charges on its surface will be dissipated over more lipids. The actual protein surface area exposed to the lipid will depend on whether the 26 kDa BR polypeptides are organized into trimers or exist as independent monomers within the DMPC bilayers at temperatures above T_m. It is suggested that each BR monomer presents at least five positive charges to the phosphocholine headgroups at the protein/lipid interface near the bilayer surface.

The positive charges presented by BR to the phospholipid headgroups may arise from basic amino acid residues or from metal ions or from both. Extensively washed PM contains approximately four to five tightly bound divalent cations (one Ca^{2+}, and three or four Mg^{2+} ions) per BR molecule [35]. The calculated apparent dissociation constant of calcium and PM is estimated to be about 10^{-8} M [35]. ^{31}P-NMR studies of PM [26] suggest that in PM, the negatively charged endogenous PM phospholipids are involved in cation binding. Thus, in the DMPC/BR vesicles used in this study, where all the endogenous PM phospholipids have been removed, there may be less than four cations per BR molecule and metal cations may not contribute significantly to the positive charge experienced by the phosphocholine headgroups. From the alignment of the amino acid sequence of the BR polypeptide with respect to the positions of the seven transmembrane α-helices in the three dimensional electron density map

[3], it would appear that each of the seven α-helices has basic amino acid residues, arginine or lysine, positioned either at the ends of the α-helical sections or in the extra-membrane segments within one or two residues of the end of the α-helix. Thus, for example, helix G has two arginine residues and helix B two lysine residues at the end of the helical section on the cytoplasmic side of the membrane. Furthermore, the three-dimensional electron density map [3] implies Lys-172 and Arg-175 on helix F are directed outwards from the centre of the BR molecule and are presumed to be able to reach the polar lipid headgroup region. These basic residues on the surface of the BR molecule at the lipid/protein interface may interact with negatively charged phosphate and sulphate groups of the endogenous PM polar lipids (i.e. glycolipid sulphates, DPh-PGS and DPhPGP [4]). Similar approaches to the study of phospholipid headgroups using deuterium NMR have shown that charged residues of well-defined and located peptides also perturb the surface of phospholipid bilayers [36,37] in a similar way to that shown here with BR as an example of a natural integral protein affecting surface charge of bilayers.

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